Sperm Precedence in Colorado Potato Beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae): Temporal Variation Assessed by Neutral Markers

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ABSTRACT Variation in sperm precedence was examined in the Colorado potato beetle, *Leptinotarsa decemlineata* Say, using neutral allozyme markers. Two temporal components were studied: (1) pairing duration and (2) time since initial pairing. We used two pairing protocols that differed in the time males and females were allowed to mate for first and second pairings. When pairings were limited to 8 h each for the first and second male, the proportion of offspring in the xth egg mass attributable to the second male, *P2*(x), rose from 68% in the first egg mass after pairing with the second male to 78% in the tenth egg mass. When pairing was extended so that the first male-female pair had time to produce three egg masses and the second male was allowed to remain with the female indefinitely, *P2*(x) rose from 44% in the first egg mass after pairing with the second male to nearly 100% in the third and subsequent egg masses. Observations that (1) multiple matings are necessary to fill the female's spermatheca and (2) the last male accounts for a greater proportion of offspring with time, provide evidence for both the sperm mixing and sperm displacement hypotheses. These data also help to explain male-guarding behavior in Colorado potato beetle. Because the allozyme markers were shown to be neutral with respect to fitness, other aspects of mating behavior could be tested by establishing a similar series of unique lines.

KEY WORDS sperm competition, electrophoresis, gene flow

THE COLORADO POTATO BEETLE, *Leptinotarsa decemlineata* Say, is polyandrous (Szentesi 1985, Ridley 1989), and because each female can mate with multiple males, the opportunity exists for sperm competition (Parker 1970, Walker 1980, Thornhill and Alcock 1983, Smith 1984, Ridley 1989, Cook et al. 1997). Possible outcomes of multiple inseminations include random mixing of sperm, first-male sperm precedence, and last-male sperm precedence. Of these, last-male sperm precedence is the most common in insects (Schlager 1960, Parker 1970, Eady and Tubman 1996, Simmons 2001), although not universal in insects or other arthropods (Watson 1991, Lorch et al. 1993, Zeh and Zeh 1994, Simmons 2001). Usually, sperm precedence is summarized for a species by the parameter *P*2, or the proportion of offspring attributable to the second male (Parker et al. 1990, Cook et al. 1997), with little attention to variation among females or within females over time (Ueno and Ito 1992, Simmons 2001). Here, we use neutral genetic markers to investigate individual and temporal variation of sperm precedence in Colorado potato beetle. This work is motivated largely by observations of mate guarding behavior by males (Szentesi 1985) and by evidence that strategies designed to delay the onset of resistance by Colorado potato beetle to transgenic plants may hinge on mating behavior and sperm precedence (Ferro 1993, Gould et al. 1994, Follett et al. 1996, Dively et al. 1998).

A myriad of structural and behavioral mechanisms can facilitate sperm competition, including multiple inseminations, stratification of sperm, direct sperm removal, sperm displacement (or "flushing"), transfer of materials to the female that cause nonreceptivity, mate guarding, and prolonged copulation (Thornhill and Alcock 1983, Simmons 2001). Stratification occurs when the last male's sperm lies over or in front of the sperm of earlier males and the principle of last-in/first-out operates. Direct sperm removal in some species occurs when males directly remove the sperm of previous males with their aedeagus as they deposit their own sperm (Waage 1979). Transfer of materials to the female that causes nonreceptivity include accessory gland donations (e.g., *Drosophila melano-*

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gaster), or a plug that is left behind at withdrawal preventing later inseminations (e.g., migratory locust, drosophilid fruit flies, honey bees) (Thornhill and Alcock 1983). Males may also prevent further matings by physically remaining close to the female. If complete sperm precedence prevails, each egg batch will have low genetic diversity, but multiple matings will increase lifetime genetic diversity. Maximal genetic diversity within an egg batch will result if sperm mixes completely after each successive insemination, producing an equal contribution from all males (Walker 1980).

The behavior of the Colorado potato beetle may give some clues as to expected patterns of sperm precedence. First, females mate frequently, which is often associated with a high degree of sperm displacement (Ridley 1989). In the laboratory, Colorado potato beetle females have been observed to copulate ≈20 times in two months (Szentesi 1985). Multiple matings may serve to replenish depleted sperm stores and maximize reproductive effort (McCauley and Reilly 1984). For example, Colorado potato beetle females mated three times have been shown to be three times more fecund than those mated only once (Szentesi 1985), and the average daily fecundity of beetles mated 4–9 times is significantly higher than beetles mated only twice (Boiteau 1988). Secondly, although copulation is completed in a relatively short time in Colorado potato beetle, on the order of 5–10 min, males can be seen holding or riding females for longer periods, which suggests males may be guarding females from other male encounters. This interpretation is reinforced by observations of aggressive attacks by resident males toward intruders, which residents win 90% of the time (Szentesi 1985, Boiteau 1988). Mate guarding behavior is also commonly associated with last male sperm precedence (Parker 1970, Thornhill and Alcock 1983, Alcock 1994).

Boiteau (1988) conducted the sole study of sperm precedence in Colorado potato beetle to date. Using two mutant color morphs to determine paternity (Boiteau 1985), two experiments were conducted: in the first experiment, a single mating by a male of one morph was followed by a single mating with a male of the other morph, and in the second experiment, the first male morph was mated 4–9 times with a female followed by a single mating with a male of the second morph. Sperm from the second male took precedence over the first male in fertilizing 53% of the eggs in the first experiment and 32% of the eggs in the second experiment (Boiteau 1988).

In the current study we extend Boiteau's work by using neutral allozyme markers to ask whether sperm precedence varies temporally given different mating conditions, specifically short- and long-duration pairings. The answers to this question can provide insight into the evolution of mate guarding behavior in this species as well as contribute to the design of strategies to delay resistance to insecticides including those incorporated into transgenic plants.

Materials and Methods

Allozyme Electrophoresis. The Colorado potato beetle strains used in this study consisted of two homozygous allelic forms, here denoted as AA and BB, of phosphoglucomutase (PGM), E.C. 2.7.5.1. PGM was chosen for genotype identification because in Colorado potato beetle it has two electromorphs at high frequency that are easily scored as homozygotes and heterozygotes. Additional enzymes also satisfy these criteria in Colorado potato beetle (Coll et al. 1994). The head and thorax of each beetle were homogenized in 30 ml of distilled water and centrifuged at 4°C for 5 min. (6,000 rpm). Electrophoresis was performed using cellulose acetate gels available from Helena Laboratories (Beaumont, TX), a method which is well suited for electrophoresis of small samples (Richardson et al. 1986, Hebert and Beaton 1989, Coll et al. 1994, Yang 1994). Gels were run in a tris-maleate-EDTA-MgCl2 (0.05 M, pH 7.8) buffer (Richardson et al. 1986) at 4°C and 180 v for 90 min. The staining recipe for PGM was unmodified from Hebert and Beaton (1989). Electromorphs were labeled such that "A" was the faster running allele and "B" the slower, running ≈0.93 the rate of "A."

Development of Allele Specific Strains. The laboratory population had been derived one generation earlier from a potato field (Ward Farm) in Stockton Co., MD. The relative frequencies of "A" and "B" alleles in this population in the field are 0.68 and 0.32, respectively (Roderick and Follett, unpublished data). During the experiments, beetles were reared on whole potato plants in large environmental chambers at 23 ± 3°C and 16:8 L:D (Everich 1994, Gilotti 1994). To ensure virginity, newly emerged second generation adults were isolated daily. A right middle leg was removed from each beetle at ≈5 d after emergence; there appeared to be no detrimental effects to the beetle following dismemberment. Leg tissue was assayed for its PGM genotype using electrophoresis. Male and female beetles that were either homozygous AA or BB were placed into two separate cages by genotype and allowed to mate and reproduce. In the subsequent generation, individual adults from each genotype cage were isolated immediately after emergence and kept separated until the beginning of each of three mating protocols. In this and subsequent procedures, male and female beetles were fed fresh potato (cultivar 'Kennebec') foliage, which was replenished daily. Vouchers of the beetles used in this study have been deposited in the Essig Museum of Entomology, University of California, Berkeley, CA.

Relative Fitness of Different Genotypes. Relative fitness of genotypes was examined in two ways. First, an experiment was designed to test for possible differences in egg production resulting from crosses of the different genotypes. Adults were weighed before mating. In this design, 32 females ranging from 7–14 d old were randomly assigned to plastic test boxes (Freeman & Sons, Inc., PCA 9394). Males, also 7–14 d old, were randomly assigned to females to create each of the possible dam/sire combinations: AAxAA,

Table 1. ANOVAs on fitnesses of PGM genotypes, measured as (A) egg output per female in the first five days of egg laying and (B) adult body mass

Source	SS ($\times 10^4$)	DF	*F*-Ratio	*P*<
A. Egg output				
Dam genotype	0.485	1	0.067	0.80
Sire genotype	2.796	1	0.387	0.54
Dam × sire genotypes	3.198	1	0.442	0.51
Error	195.2	27		
B. Adult Body Mass				
Genotype	5.22	1	1.33	0.25
Sex	53.38	1	13.61	0.001
Genotype × Sex	1.69	1	0.43	0.5
Error	478.3	122		

AAxBB, BBxAA, and BBxBB (n = 8, 8, 6, 9, respectively). The males were left with the females for 8 h then removed. Ten hours later a second male of the same genotype as the first was placed with the females for 8 h then removed. Females were left alone to lay eggs and egg masses were checked and removed daily. The number of eggs was counted for each egg mass laid and compared across all four mating combinations noted above. Mating frequency during the two 8 h pairings may have influenced subsequent female fecundity but was not determined. Second, the adult body weight of beetles of each sex was compared between AA and BB strains.

Statistics of Sperm Precedence. In polyandrous organisms, *P*2 is the statistic describing sperm utilization and is equal to the proportion of offspring attributable to the second of two competing males (Parker et al. 1990, Cook et al. 1997). *P*2 is typically a single statistic based on the overall outcome of sperm utilization for a group of individuals. In animals that have distinct egg batches or clutches, as many insects do, *P*2 can be calculated for each of these cohorts, giving what we term "partial" *P*2, or *P*2(x), where x is the clutch number in a series of clutches. Sperm precedence was summarized for each egg mass as *P*2(x). All statistical comparisons were performed on arcsine transformed *P*2(x)'s.

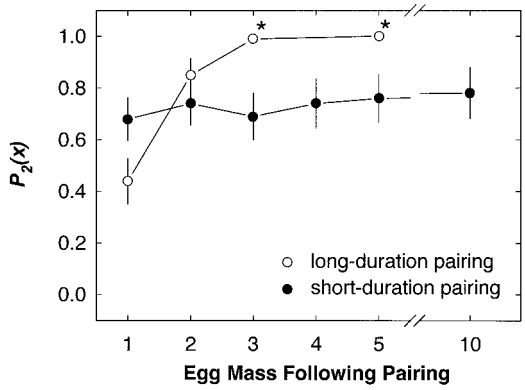

Fig. 1. Proportion of offspring attributable to the second male, *P*2(x), under short-duration (closed circles) and long-duration pairings (open circle). Standard errors calculated from raw data are shown except where smaller than symbol. The change in *P*2(x) from egg mass one to egg mass five differed significantly between the two protocols (repeated measures ANOVA following arcsine transformation; $F = 14.5$, df = 3, 69, $P < 0.001$; see text). *P*2(x) was significantly greater ($P < 0.05$) under long-duration pairings for egg masses three and five (following arcsine transformation; dates denoted in figure by *).

Effect of Duration of Pairing. Two pairing protocols were compared with test for the effects of (1) short-duration pairings, and (2) long-duration pairings. This design was used, rather than comparing successive copulations by two different males, to better simulate situations in natural populations.

In the short-duration protocol, the pairing procedure was similar to that above and was examined in two parts. In the first, 10 "BB" females (dams 1–10) were chosen randomly and each was paired first with a randomly chosen "BB" male for 8 h, then no male for 10 h, and then paired with a randomly chosen "AA" male for 8 h. In the second, nine "BB" females (dams 11–19) were chosen randomly and each was paired first with a randomly chosen "AA" male for 8 h, then no male for 10 h, and then paired with a randomly chosen "BB" male for 8 h. Females were allowed to lay eggs as before. Egg masses were collected and counted daily. Larvae were reared from individual egg masses to second instar in 9 cm petri dishes with moistened filter paper and excised potato leaflets in an incubator at 24°C (Everich 1994, Gilotti 1994). Individual larvae were then frozen at −78°C until used in electrophoresis. For each egg mass, 10 individuals were assayed individually by electrophoresis for genotype. *P*2(x) was recorded for egg masses numbering 1, 2, 3, 4, 5, and 10 following pairing with the second male. Where possible, one egg mass was assayed numbering between 12 and 15 [*P*2(12–15)]. After the experiment, electrophoretic analysis was performed on all adults to confirm their PGM genotype.

In the long-duration protocol, five "AA" females were chosen randomly and each was paired first with a randomly chosen "AA" male until three egg masses were laid. At this point the first male was removed and replaced with a randomly chosen "BB" male until the end of the experiment (15 d). Simultaneously, five "AA" females were chosen randomly and each was paired first with a randomly chosen "BB" male until

Table 2. *P2*(x) values by dam and egg mass scored following pairing with second male for (A) short-duration pairings and (B) long-duration pairings. Families are sorted by total *P2*. Missing values (egg mass laid but not scored electrophoretically) denoted by period.

Dam	A. Short-duration pairings eggmass							Total *P2*
	1	2	3	4	5	10	12–15	
11	0.00	0.00	0.00	.	0.00	0.00	0.00	0.00
15	0.00	0.00	0.00	0.00	0.10	0.00	0.08	0.03
17	0.00	0.00	0.00	.	0.10	0.10	.	0.04
10	0.40	0.70	0.40	0.40	0.60	0.20	.	0.45
12	1.00	0.67	0.50	0.40	.	.	.	0.64
13	0.50	0.60	0.20	0.80	1.00	1.00	.	0.68
14	0.70	0.70	0.50	0.56	0.90	1.00	.	0.73
16	0.60	0.70	0.70	0.90	1.00	0.90	.	0.80
5	0.75	0.80	0.90	0.90	0.60	1.00	1.00	0.85
8	0.80	0.80	1.00	0.90	0.80	1.00	.	0.88
6	0.60	1.00	1.00	1.00	1.00	1.00	.	0.93
3	0.63	1.00	1.00	1.00	1.00	1.00	1.00	0.95
19	1.00	1.00	0.90	1.00	.	1.00	.	0.98
1	1.00	1.00	1.00	.	.	.	.	1.00
2	1.00	1.00	1.00	1.00	1.00	1.00	.	1.00
4	1.00	1.00	1.00	.	1.00	1.00	.	1.00
7	1.00	1.00	1.00	.	1.00	1.00	.	1.00
9	1.00	1.00	1.00	.	1.00	1.00	1.00	1.00
18	1.00	1.00	1.00	.	1.00	1.00	.	1.00

Dam	B. Long-duration pairings eggmass				Total *P2*
	1	2	3	5	
10	0.00	0.40	1.00	1.00	0.60
8	0.50	0.89	.	.	0.69
6	0.20	0.60	1.00	1.00	0.70
2	0.20	0.80	0.90	1.00	0.73
7	0.20	1.00	1.00	1.00	0.80
1	0.50	1.00	1.00	1.00	0.88
4	0.60	0.90	1.00	1.00	0.88
5	0.60	1.00	1.00	1.00	0.90
3	0.70	1.00	1.00	1.00	0.93
9	0.89	0.90	1.00	1.00	0.95

three egg masses were laid, then the first male was removed and replaced with a randomly chosen "AA" male until the end of the experiment. *P2*(x) was recorded for egg masses numbering 1, 2, 3, and 5 following pairing with the second male. *P2*(x) was compared between the short-duration pairings and the long-duration pairings for egg masses 1, 2, 3, and 5. Adult genotypes were confirmed by electrophoresis.

Results

Relative Fitness of Different Genotypes. There was no evidence of a difference in fitness between the genotypes used in this study. Egg output per female averaged 211 ± 14 eggs (mean ± SE) in the first five days of egg laying and did not differ between dam genotypes (AA versus BB), sire genotypes (AA versus BB), nor the interaction of dam and sire genotypes (Table 1). Adult body weight did not differ significantly between AA and BB genotypes nor was there a genotype by sex interaction (Table 1). As expected, female beetles were significantly larger than males (147 ± 3 mg and 119 ± 2 mg, respectively).

Effect of Duration of Pairing. In the short-duration pairings the proportion of offspring attributable to the second male, *P2*(x), did not differ significantly over the first 3 d [repeated measures analysis of variance (ANOVA) following arcsine transformation, $F = 0.57$, df = 2, 36, $P < 0.56$] but did increase significantly by day 10 ($F = 3, 78$, df = 4, 60, $P < 0.008$; Fig. 1). *P2*(1) was 0.68 (±0.08 SE) in the first egg mass after the second male pairing. *P2*(x) increased slightly to 0.78 (±0.10) by the tenth egg mass but dropped to 0.62 (±0.23) for later egg masses, *P2*(12–15) (not shown in Fig. 1). In the long-duration pairings *P2*(x) did increase significantly over the first 3 d (repeated measures ANOVA following arcsine transformation, $F = 32.5$, df = 2,16, $P < 0.001$; Fig. 1). *P2*(x) was initially low with a mean of 0.44 (±0.09) in the first egg mass after the second male was introduced but increased rapidly to 0.99 (±0.01) by the third egg mass and 1.00 (±0.0) by the fifth egg mass.

Duration of pairing did have a significant effect on proportion of offspring attributable to the second male, *P2*(x) (Fig. 1). The change in *P2*(x) from egg mass one to egg mass five differed significantly between the two protocols (repeated measures ANOVA following arcsine transformation; $F = 14.5$, df = 3, 69, $P < 0.001$). The long-duration pairings had a steeper initial slope reaching nearly 100% by the third egg mass. *P2*(x) for egg masses three and greater were significantly greater for the long-duration pairings than for the short duration pairings ($t = 2.29$, df = 26, $P < 0.03$ and $t = -2.13$, df = 23, $P < 0.04$, respectively

for egg masses three and 5); comparisons between earlier egg masses were not significant ($P > 0.05$).

Three of the females used in the short duration pairings (dams 11, 15, and 17) used little of the second male's sperm in contrast to the others females used in these pairings. We do not know why this difference among dams (or sires) in the short duration pairings occurred, and it may be simply random variation in the time before individuals are ready to mate. The females and males used in the study were all of equal age and reared under the same conditions. When these three females were removed from the analysis, the results were qualitatively the same as reported above. Also, six of the males in the short duration manipulation achieved and overall *P2* of 1.0, while the highest overall *P2* for males in the long duration manipulation was 0.95.

Discussion

Neutral markers facilitate the assessment of sperm precedence in the Colorado potato beetle and other insects. Evidence presented here as well as population studies (Coll et al. 1994) demonstrates no selective advantage to either of the two alleles at the PGM locus in Colorado potato beetle. While this study exploited just two distinct genotypes, the variability at other allozyme loci and other genetic markers in Colorado potato beetle makes possible much more elaborate experiments using neutral markers (Coll et al. 1994, Azeredo-Espin et al. 1996, Roderick 1996).

Female fecundity averaged 211 ± 14 eggs over the first five days of egg laying, and there was no significant difference among crosses (Table 1). This rate is comparable to oviposition reported elsewhere for Colorado potato beetle (Peferoen et al. 1981). The "black" and "white" mutant strains previously used to study sperm precedence in Colorado potato beetle were considerably less fit when mated *inter se* compared with a "normal" strain (Boiteau 1988). When the mutant strains were crossed, fitness, as measured by fertility, improved to equal that of the "normal" strain. However, whether all genotypes produced by crossing the two strains were equally fit is unknown. The use of presumptive neutral allozyme markers from field-collected beetles reduces the chance for fitness problems that are sometimes associated with mutant strains.

The sperm stratification hypothesis for sperm competition predicts that $P2(x)$ would decrease over time as recently deposited sperm is depleted through use, and that sperm stored from earlier copulations will have an unobstructed path to the aperture of the spermatheca. We observed an increase in $P2(x)$ over time in the short duration and long duration pairings; strictly speaking this is contrary to the stratification model, although to an extent some diffuse stratification may be operating to produce the incomplete sperm precedence we observed.

Several other mechanisms of sperm competition could be acting to produce the pattern of sperm precedence observed in our experiments. Long duration matings resulted in the elimination of first-male sperm within three egg masses (Fig. 1). With short duration pairings, sperm use was comparable to that of the long duration pairings after one egg mass (68 ± 8% versus 43 ± 8% and not significantly different), but $P2(x)$ values soon leveled off at ≈75%, suggesting sperm were mixing and reached an equilibrium value. The incomplete precedence of second-male sperm with short-duration pairings suggests first male sperm is somehow depleted when the second male mates or flushed out by the second male during copulation. If sperm is being depleted, we would expect considerable variation among clutches in sperm precedence, because different time intervals will have elapsed between when second mates introduce sperm and the previous copulation or oviposition event. However, variation in *P2* did not change through the short duration experiment.

Sperm precedence could result from leakage of sperm from the spermatheca between matings. In this case, sperm from the most recent mating is more prevalent because a portion of the sperm stored earlier has leaked out of the spermatheca and is no longer useful for fertilization. Flushing of part of the sperm in the spermatheca will have this result also. Evidence for degradation of sperm would be an increase in $P2(x)$ over time in the short duration treatment. In our study, the slight increase in $P2(x)$ over time was not significant. If sperm become less competitive over time while stored in the spermatheca, following type II or III survivorship curves (Price 1984), an increasing proportion of eggs might be fertilized by the second-male sperm with each successive egg mass. We found no evidence for this effect.

The high degree of sperm precedence is consistent with theoretical considerations regarding mate guarding (Alcock 1994). Three matings are required in Colorado potato beetle to fill the spermatheca (Boiteau 1988), and the more copulations accrued by the male, the greater number of eggs he will fertilize. Therefore, male-guarding behavior serves two purposes: the male chaperon can mate with the female more than once increasing reproductive output, and females cannot mate with another male, thus ensuring fertilization of those eggs. Long bouts of guarding increase the likelihood that the female will deposit an egg mass with eggs fertilized largely by the attending male. Both mating experiments demonstrate that sperm precedence is incomplete. Further, long duration mating experiment indicates that multiple copulations in Colorado potato beetle can cause total sperm displacement. These observations will be of value in designing strategies for the control of Colorado potato beetle that rely on the effects of multiple mating.

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